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Physical characteristics of the cerebral big prothoracicotropic hormone from *Manduca sexta*

D. P. Muehleisen*, E. J. Katahira, R. S. Gray and W. E. Bollenbacher

Department of Biology, CB # 3280, Coker Hall 010A, University of North Carolina at Chapel Hill, Chapel Hill, (North Carolina 27599-3280, USA)
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Abstract. The prothoracicotropic hormones (PTTHs) are cerebral peptides that control insect postembryonic development by stimulating the prothoracic glands to synthesize ecdysteroids. In *Manduca sexta*, the tobacco hornworm, two classes of PTTH are distinguished by their M_r, small (ca. 7 kDa) and big PTTH (ca. 25–30 kDa). Little is known about the physical nature of the PTTHs and this study takes a first step towards defining characteristics of the *Manduca* big PTTH. The neurohormone has a Stokes radius of 2.59 nm and a sedimentation coefficient of 2.76 S. Based on these data, an M_r of 29,443.7 and an f/f_0 of 1.27 were calculated. Combined, the physical data reveal *Manduca* big PTTH is an asymmetrical acidic homodimeric peptide with intra- and intermolecular disulfide bonds.
Key words. Prothoracicotropic hormone; *Manduca sexta*; anti-PTTH monoclonal antibody; sedimentation coefficient; frictional ratio; molecular weight.

Insect postembryonic development is directed through the precise temporal regulation of the release of peptide neurohormones produced by specific cerebral neurosecretory cells (NSC)[1]. These peptides control development by modulating the activity of the sesquiterpenoid juvenile hormones and the polyhydroxylated ketosteroid ecdysteroids. Quantitative and qualitative fluctuations in the hemolymph (blood) titers of these two hormone families determine both the timing and the character of an insect's molt, i.e., larval or metamorphic[2]. Ecdysteroid biosynthesis by the prothoracic glands is regulated by the cerebral peptide prothoracicotropic hormone (PTTH)[3]. In the tobacco hornworm, *Manduca sexta*, PTTH appears to exist in two forms of acidic proteins, big ca. 25–30 kDa[4,5] and small ca. 7 kDa[5], and each appears to have molecular variants[3,5,6]. An immunoaffinity chromatography-based purification of the big PTTH has yielded three peptides, which by sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) under non-reducing (minus β-mercaptoethanol, βME) conditions have relative molecular masses (M_r) of ca. 25.5, 24.5, and 23 kDa. The variants appear to be dimers consisting of a ca. 16 kDa peptide[7]. This dimeric configuration of identical or near identical subunits is similar to that determined for the PTTH from the commercial silk moth, *Bombyx mori*[27]. However, a partial sequence of the *Manduca* 16 kDa monomer has not revealed any sequence homology with the *Bombyx* PTTH, and the *Bombyx* PTTH does not have PTTH activity in *Manduca*[7]. The partial sequence data obtained has revealed some sequence similarity to the vertebrate cellular retinoid binding proteins, cellular retinoic acid and cellular retinol binding proteins, CRABP and CRBP respectively, members of the nerve myelin P2 protein superfamily[8]. Like other proteins of the P2 superfamily, big PTTH is an acidic protein[5,7,8], pI 5.2, having a high glutamic acid and glycine content and monomer size comparable to CRBP and CRABP[7].

Big PTTH is produced by only a few cerebral peptidergic neurons[6] and is not available in sufficient quantity for direct analysis of its subunit conformation. To circumvent this obstacle, the peptide's native structure in relation to its monomer has been investigated indirectly using the methods developed by Siegel and Monty[10] for partially purified peptides. This report details the results of this analysis, which use independently determined Stokes radius (R_s) and sedimentation coefficient ($S_{20,w}$), to achieve a more precise estimate of the dimeric peptide's mass and a prediction of its shape.

Materials and methods

Animals. Manduca sexta larvae were reared on an artificial diet[10] under non-diapause conditions (LD 16:8) at 26 °C and 60% RH. Pupae were staged at ecdysis, which was designated as day 0.

Chemicals and reagents. α[23,24-^{3}H(N)]-Ecdysone (81.9 Ci mmol^{-1}) was from New England Nuclear Corp. (Boston, MA) and ecdysone was a gift from D. H. S. Horn (Melbourne, Victoria, Australia). Acrylamide, piperazine diacrylamide, β-mercaptoethanol (βME), Tris-(hydroxymethyl)aminomethane, and glycine were from Bio-Rad Laboratories (Richmond, CA). Immobilon™-P transfer membranes were from Millipore Corp. (Bedford, MA), HPLC grade acetonitrile was from Mallinckrodt Inc. (Paris, KY), enzyme grade sucrose

was from Life Technologies Inc. (Gaithersburg, MD), and trifluoroacetic acid (TFA) was from Sigma Chemical Co. (St. Louis, MO).

Big PTTH assays. In vitro bioassay: Big PTTH bioactivity was detected and quantified using the in vitro day 0 pupal prothoracic gland (PG) bioassay for the neurohormone[11]. PTTH-stimulated PG ecdysteroid synthesis was quantified by an ecdysone radioimmunoassay as described previously[6]. For the in vitro assay, one gland of a pair was an experimental and the other a control. Stimulation of the experimental PG was noted as an activation ratio (A_r), which is the amount of ecdysteroid synthesized by the experimental gland divided by the amount synthesized by the control gland. A PTTH unit (U) is the amount of the neurohormone that half maximally stimulates a PG.

Enzyme linked immunosorbant assay (ELISA): Size exclusion high performance liquid chromatography (SE-HPLC) and sucrose density gradient centrifugation fractions were assayed for big PTTH by ELISA using an affinity purified anti-big PTTH monoclonal antibody (big PTTH A2H5 MAb) as described previously[6], except for elimination of the amplification step.

Separation of big PTTH. Partially purified big PTTH used for analysis was a C18-HPLC enriched preparation prepared as described previously[6].

SDS-PAGE and Western blotting: SDS-PAGE was performed as previously described[12], with the exception of the separating gel being a 9.5–16% exponential acrylamide gradient. Gels were either silver stained[13] or electro-transferred onto Immobilon™ as described previously[7]. Western blots were immunostained using the big PTTH A2H5 MAb, an alkaline phosphatase labeled secondary antibody (Bio-Rad), and 5-bromo-4-chloro-3-indolyl phosphate and nitroblue tetrazolium (Bio-Rad) as enzyme substrates[7].

Size exclusion chromatography: Size separation of partially purified big PTTH was performed using an Ultropac TSK-G2000SW HPLC column (7.5 × 600 mm, Pharmacia-LKB, Piscataway, NJ) on a 2150 LKB HPLC with a gradient solvent delivery system and 2140 Rapid Spectral Detector (Pharmacia-LKB). Fractions (1 ml) were collected (2212 Helirac, Pharmacia-LKB) and aliquots of each fraction assayed for big PTTH. The R_s values for the protein standards used are: bovine serum albumin, 3.6 nm[14]; carbonic anhydrase, 2.4 nm[15]; cytochrome C, 1.66 nm[16]; ferritin, 6.1 nm[17]; myoglobin, 1.9 nm[18]; and ovalbumin, 2.7 nm[19]. Partition coefficients (K_{av}) were calculated as described previously[20], and big PTTH's R_s was determined from a plot of $(-\log K_{av})^{1/2}$ vs. R_s of protein standards. The M_r and frictional ratio (f/f_0) were determined by the method of Siegel and Monty[9], and a partial specific volume of 0.725 $cm^3\ g^{-1}$ was used in calculating the M_r[18]. The axial ratio (a/b) of a hydrodynamically equivalent prolate ellipsoid was determined from the f/f_0[21,22].

Sucrose density gradient centrifugation: HPLC enriched big PTTH (800 U mg^{-1}) was fractionated on a sucrose gradient by centrifugation with a SW-65Ti rotor (Beckman, Palo Alto, CA). Varying amounts of big PTTH (100 U to 400 U) in phosphate buffer (0.05 M, 0.15 M KCl, pH 6.8) were layered onto a 10 to 30% linear sucrose gradient in the same buffer and centrifuged at $2 \times 10^5 \times g$ for 16 h. The sucrose concentration in collected fractions (0.2 ml) was determined using a refractometer and the sucrose then removed by Ultrafree®-MC filters (10,000 NMWL: Millipore, Bedford, MA) with each fraction washed 3 times with HPLC grade water. Aliquots of each fraction were assayed for PTTH by ELISA. The sedimentation coefficient ($S_{20,w}$) for ELISA positive fractions was determined[23] and verified[24]. [^{14}C]-Carbonic anhydrase (3.2 S) (Amersham, Chicago, IL) was added to each gradient as an internal standard.

Results

Big PTTH M_r by SDS-PAGE. A Western blot of the C18-HPLC enriched big PTTH, run on SDS-PAGE under non-reducing conditions (minus βME), revealed 3 immunoreactive bands migrating at ca. 25.5, 24.5, and 23 kDa (fig. 1, lane 1) as previously described. Under reduced conditions (plus βME) no immunoreactive bands were evident (fig. 1, lane 2). Silver stained, re-

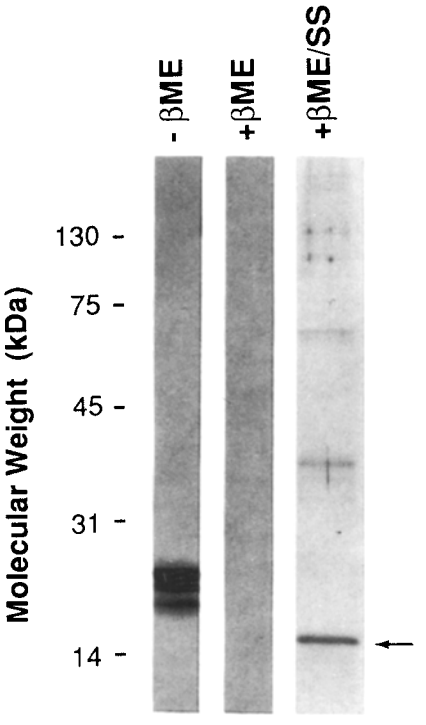

Figure 1. Western blots of SDS-PAGE separated C18-HPLC enriched big PTTH under non-reduced ($-\beta$ME) and reduced ($+\beta$ME) conditions subjected to immunostaining with the big PTTH A2H5 MAb or SDS-PAGE silver staining ($+\beta$ME/SS) to characterize the protein's size and subunit composition.

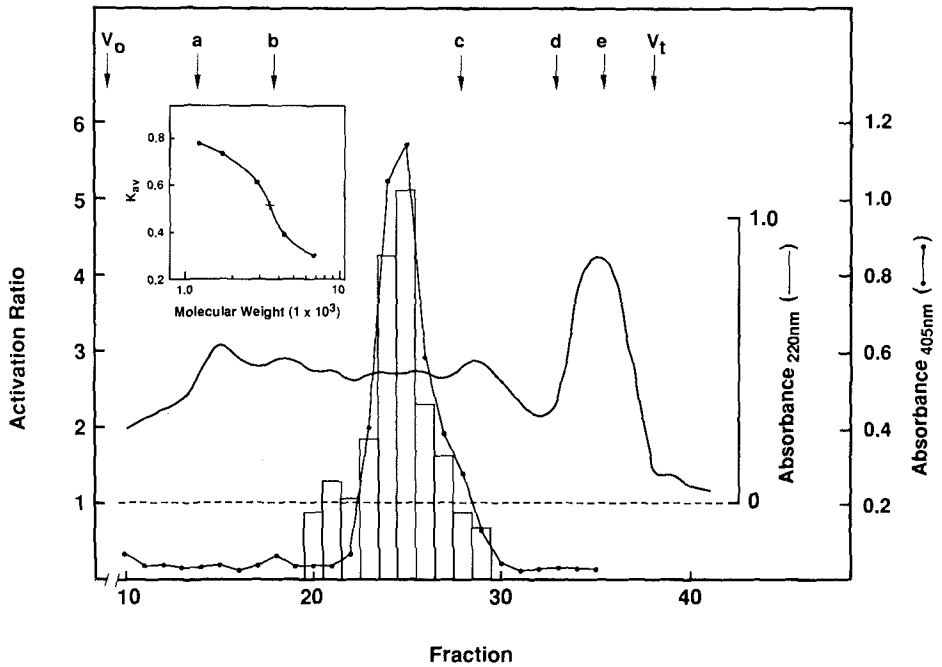

Figure 2. SE-HPLC fractionation of C18-HPLC enriched big PTTH screened by ELISA and in vitro PG bioassay to determine the protein's M_r. Inset: K_{av} plot of protein standards to determine big PTTH's M_r. V_o, ferritin; a, bovine serum albumin; b, ovalbumin; c, carbonic anhydrase; d, myoglobin; e, cytochrome C; and V_t, potassium dichromate.

duced SDS-PAGE separated big PTTH revealed the single ca. 16 kDa band (fig. 1, lane 3), which appears to be the big PTTH monomer[7]. The faint 60 and 40 kDa bands appear to be antibody that has leached off the affinity column, since on Western blots of the affinity purified big PTTH an anti-IgG secondary antibody recognizes these bands with or without primary antibody.

M_r by HPLC and Stokes radius determination. To determine if the molecular weight of the mature big PTTH protein is larger than that suggested by SDS-PAGE and thus more consistent with that predicted by the ca. 16 kDa monomer, the peptide's M_r was determined by SE-HPLC (fig. 2). Big PTTH elution, determined by ELISA and in vitro bioassay, occurred as a single, symmetrical peak. Big PTTH's M_r by K_{av} plot (fig. 2 inset) was determined to be ca. 33 kDa, which approximates a previous chromatography-based estimate[5], and is consistent with that for a dimeric protein composed of ca. 16 kDa monomers[7].

Proteins do not always migrate relative to the log of their molecular weight by size exclusion chromatography. The molecule's R_s is a more accurate means of determining molecular mass[20]. Big PTTH's R_s was determined according to the procedure of Siegel and Monty[10]. From the linear relationship of the R_s for known proteins versus $(-\log K_{av})^{1/2}$ (ref. 20), big PTTH's R_s was determined to be 2.59 nm (fig. 3). This R_s was verified using data previously obtained by standard gel filtration chromatography with Sephadex G-50 (data not shown)[5].

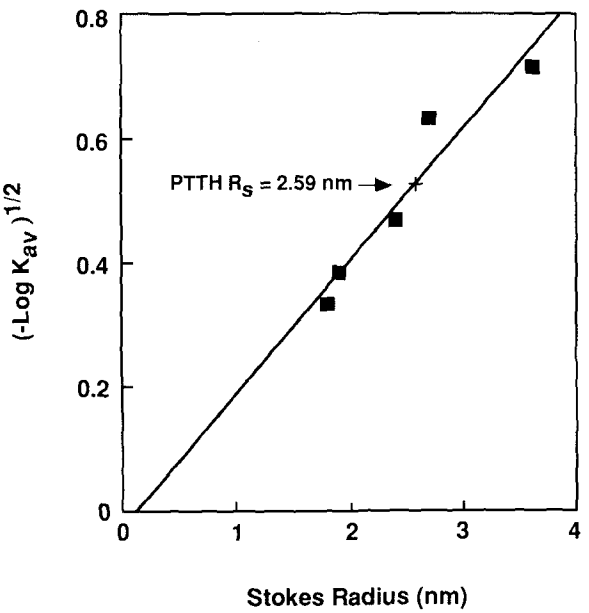

Figure 3. Stokes radius determination of *Manduca* big PTTH.

Sedimentation coefficient. To gain insight into PTTH's shape relative to its mass, the neurohormone was fractionated by sucrose density gradient centrifugation. A big PTTH ELISA positive peak was obtained (fig. 4), and calculation of the protein's sedimentation coefficient[23] yielded a value of 2.76 S, which is consistent with a protein of ca. 30 kDa.

Molecular weight determination. The SE-HPLC and sedimentation coefficient data were used to estimate

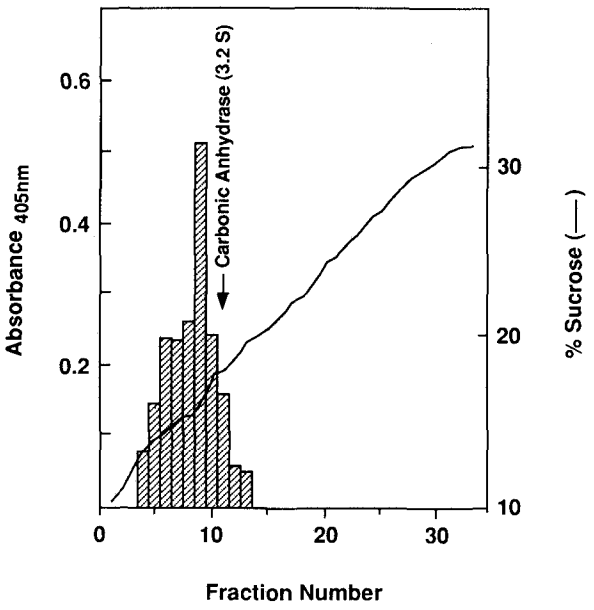

Figure 4. Sucrose density gradient centrifugation of C18-HPLC enriched big PTTH with identification of the protein by ELISA.

Molecular properties of biologically active *Manduca sexta* big PTTH isolated from day 1 pupal brains

Physical parameter	Value
Sedimentation coefficient (S_{20w})	2.76 ± 0.061 (5)*
Stokes radius (nm)	2.59 ± 0.1 (4)*
Relative molecular mass (M_r)	29,443.7
Frictional ratio (f/f_0)	1.27
Axial ratio (a/b) (prolate ellipsoid)	5.08

*Values in parentheses denote the number of replicated determinations.

more precisely big PTTH's molecular weight. The protein's R_s and $S_{20,w}$ yielded an M_r of 29,443.7 (table)[9]. The protein's frictional ratio was estimated to be 1.27. If big PTTH is a prolate ellipsoid, then it would have an axial ratio of 5.08[21,22] making the native protein highly asymmetrical.

Discussion

The physical data obtained for the *Manduca* big PTTH provides a more precise perspective on its size and conformation. The most reliable molecular mass determined for the asymmetrical big PTTH was ca. 29.5 kDa, and significantly, this mass is more in agreement with the protein's existence as a dimer of identical, or near identical, ca. 16 kDa subunits. The larger ca. 33 kDa size for the peptide determined by SE-HPLC could be a function of the protein's asymmetry, which may result in its behaving like a larger protein when fractionated by size exclusion methods. The fact that big PTTH behaves as a smaller than expected protein on SDS-PAGE under non-reducing conditions suggests the protein contains intra- as well as inter-molecular disulfide bonds[25].

While the molecular weight data support the ca. 16 kDa subunit hypothesis, they pose problems in interpreting the Western blot-derived big PTTH molecular weight from the non-reduced SDS-PAGE separation. The latter data suggest the protein exists as 3 smaller, similarly sized molecular variants, but their subunits seem to be the same ca. 16 kDa protein. Because disulfide bonds within proteins are not broken under non-reduced SDS-PAGE conditions (minus βME), big PTTH's multiple molecular weights may be due to varying degrees of intra-molecular disulfide bonding that could yield different shapes, and thus multiple apparent molecular weights[26]. In this context, the sucrose density gradient and SE-HPLC data point to big PTTH existing as a larger single protein. However, these findings must be interpreted with caution. For both studies, big PTTH activity eluted as a single symmetrical peak, indicative of a protein having a homogeneous molecular weight. Unfortunately, however, the two methods have comparatively limited resolving power for proteins with similar molecular masses, which precludes using these data to conclude that big PTTH is one protein. However, the finding that *Manduca* big PTTH is a dimer composed of a single ca. 16 kDa subunit enhances the data's efficacy.

The *Bombyx* and *Manduca* PTTHs are the only PTTHs characterized thus far. Although their physical characteristics have not been completely determined, it is clear that the two proteins, while having some similarities, are quite different. Their similarity is at the level of both proteins existing as dimers of identical or near identical units[7,27,28]. There is no known sequence similarity between the two proteins at the amino acid level[7,27], and they have very different pIs (8.9 for *Bombyx* PTTH and 5.1 for *Manduca*)[7]. These differences are further supported by the peptides not having PTTH activity in the other insect[7], which is a remarkable finding given the pivotal role PTTH plays in the insect's development. Furthermore, under reduced and non-reduced SDS-PAGE conditions, the two proteins have different apparent molecular weights[7,28]. Together, these data indicate that the two characterized insect PTTHs are chemically different from one another, despite the similarity in their organization.

Beyond this point, definitive resolution of the molecular dynamics of the *Manduca* big PTTH will depend upon having sufficient peptide for direct analysis. Given the paucity of neurohormone in each *Manduca* brain, the only reasonable means by which enough pure protein could be obtained is via expression of the big PTTH gene in an expression system that would yield processed, native protein in high quantity. The information presented here will allow the basic physical characteristics of the expressed protein to be compared to the native protein to determine if the expressed protein is processed properly.

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* To whom correspondence should be addressed.

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